Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 29, Issue of July 22, pp. 18739–18742, 1994

Startle Disease Mutations Reduce the Agonist Sensitivity of the Human Inhibitory Glycine Receptor*

(Received for publication, March 3, 1994, and in revised form, May 23, 1994)

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The receptor for the inhibitory neurotransmitter glycine is a member of the ligand-gated ion channel receptor superfamily. Point mutations in the gene encoding the α1 subunit of the glycine receptor-channel complex (GlyR) have recently been identified in pedigrees with the autosomal dominant neurological disorder, startle disease (hyperekplexia). These mutations result in the substitution of leucine or glutamine for arginine 271. This charged residue is located near the ion channel region and is predicted to affect chloride permeation through the GlyR. We found little evidence for this role from the anion/cation selectivity and lack of pronounced rectification of currents flowing through recombinant human α1 subunit GlyRs containing the startle disease mutations. We reveal, however, that the startle disease mutations profoundly disrupt GlyR function by causing 230–410-fold decreases in the sensitivity of receptor currents activated by the agonist glycine. Additionally, we report corresponding 56- and 120-fold reductions in the apparent binding affinity (K_i) of glycine to the mutant GlyRs, but no change in the binding affinity of the competitive antagonist, strychnine. Thus, startle disease reduces the efficacy of glycinergic inhibitory neurotransmission by producing GlyRs with diminished agonist responsiveness. Our results show that startle disease mutations define a novel receptor activation site.

The glycine receptor (GlyR)[1] is a member of the ligand-gated ion channel superfamily (1–3) of neurotransmitter receptors responsible for fast synaptic transmission in the nervous system. Members of this superfamily, which includes the receptors for γ-aminobutyric acid ($GABA_AR$), acetylcholine (nAChR), and 5-hydroxytryptamine, share considerable sequence and structural homology. Receptors are comprised of five subunits, arranged in the postsynaptic membrane of neurons so as to form a central, ion channel, which in the case of the GlyR is selective to Cl^-. Each subunit in turn contains a large N-terminal extracellular region and four membrane-spanning domains (M1–M4), of which the second (M2) lines the ion channel (1–3). Binding sites for glycine and other GlyR agonists, and for the competitive antagonist, the plant alkaloid strychnine, have been previously shown to be localized in the N-terminal extracellular region of α subunits (4–8).

The GlyR *in vivo* mediates inhibitory neurotransmission in the spinal cord and brain. Reduced glycinergic inhibition caused by subconvulsive strychnine poisoning results in muscular hypertonia, heightened reflex excitability, and an exaggerated response to sensory stimuli (9). These symptoms are similar to those of the autosomal dominant neurological disorder, startle disease, also known as hyperekplexia or Kok's disease (10–13). This disorder is characterized by a marked muscular hypertonia in infancy and an exaggerated startle response to unexpected sensory stimuli that persists in adulthood (10–13). The startle disease gene has been mapped to human chromosome 5q32 (13, 14), a region that contains the genes for a number of neurotransmitter receptors including that for the α1 subunit of the GlyR (15). More recently, point mutations in the gene encoding the α1 subunit of the GlyR have been identified in startle disease pedigrees (16). Reduced glycinergic inhibition arising from the impaired function of mutated GlyRs may thus underlie the symptoms of startle disease.

Two point mutations involving the same base pair in the gene encoding the α1 subunit of the GlyR have been identified in four startle disease pedigrees (16). These mutations result in the substitution of an uncharged amino acid (leucine or glutamine) for a positively charged arginine residue at position 271 (Arg-271). This residue is located at the extracellular margin of the channel-lining M2 region, suggesting that its mutation may impair ion permeation through the GlyR. We investigated the effects of the startle disease mutations on GlyR function by replacing Arg-271 with either leucine (R271L) or glutamine (R271Q) in GlyR α1 subunit cDNA using site-directed mutagenesis. Mutated cDNA or unmodified (WT) cDNA was then transfected into mammalian 293 cells resulting in the transient expression of homomeric human α1 subunit GlyRs (17, 18), which were subsequently analyzed using patch-clamp techniques and [^{3}H]strychnine binding assays. We have found that startle disease mutations caused no major alterations to ion permeation through the GlyR, but rather, dramatically reduced the receptor's agonist sensitivity. This latter finding, in addition to explaining the startle disease phenotype, identifies an unexpected structural determinant of GlyR function.

EXPERIMENTAL PROCEDURES

Expression of Mutated GlyR α1 Subunit cDNAs—Mutations in the human GlyR α1 subunit cDNA (18) were constructed by using the oligonucleotide-directed polymerase chain reaction mutagenesis method (19) and were confirmed by DNA sequencing. The single-letter code for amino acids is used to describe mutations. The letter preceding the position number refers to the amino acid in the wild type (WT) GlyR and the letter after the number refers to the amino acid replacing the WT amino acid. For example, R271L refers to the mutation of arginine at position 271 to leucine. GlyR α1 subunit cDNA was transfected, using the 3% CO_2 method (20) into exponentially growing 293 cells (21) as previously described (5). Patch-clamp studies and [^{3}H]strychnine binding assays were conducted 48 h after transfection.

Patch-Clamp Studies—Transfected cells were patch-clamped in the whole cell mode (22) while continually superfused with a bath solution

* This research was supported by the Australian National Health and Medical Research Council, the R. T. Hall Trust, and the Vincent Fairfax Family Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: GlyR, glycine receptor; $GABA_AR$, γ-aminobutyric acid type A receptor; nAChR, acetylcholine receptor; WT, wild type.

TABLE I

Mean EC_{50}, Hill coefficient (h), and I_{max} values for glycine-activated currents in 293 cells expressing WT and mutant GlyRs

The values in parentheses are the -fold changes relative to the values for the WT GlyRs. Data represent mean values with S.E. from 5–6 cells.

GlyR	EC_{50}	h	I_{max}
	mM		*nA*
WT	0.029 ± 0.004 (1)	1.6 ± 0.2 (1)	5.0 ± 2.1 (1)
R271L	6.7 ± 1.5 (230)	1.3 ± 0.1 (0.8)	5.7 ± 2.8 (1.1)
R271Q	12 ± 0.6 (410)	1.5 ± 0.1 (0.9)	1.8 ± 0.6 (0.4)

containing (in mM) 140 NaCl, 10 glucose, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, pH 7.4. Glycine dissolved in this solution was rapidly applied to the chamber using a microperfusion tube system. Voltages were corrected for junction potentials using the JPCalc program (23). The pipette solution contained (in mM) 145 CsCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 EGTA, pH 7.4. In experiments measuring reversal potentials CsCl was replaced by KCl. The EC_{50} value and Hill coefficient (h) for glycine-activated currents in individual cells were calculated by fitting data using a non-linear least squares algorithm to the Hill equation: $I/I_{max} = [G]^h/(EC_{50}{}^h + [G]^h)$, where I is the magnitude of the peak current elicited by a concentration [G] of glycine and I_{max} is the magnitude of the maximum peak current elicited by saturating concentrations of glycine.

[^{3}H]Strychnine Binding Assays—Transfected cells were incubated with [^{3}H]strychnine (1–50 nM; 24.5 Ci/mM, DuPont NEN) with and without 10 μM cold strychnine to determine nonspecific binding. After incubation to equilibrium at 4 °C for 60 min, cells were collected by rapid filtration onto Whatman GF/B filter paper and the amount of radioactive strychnine remaining bound determined. The K_d and B_{max} for the [^{3}H]strychnine saturation isotherms and IC_{50} and K_i for glycine displacement of bound [^{3}H]strychnine were estimated using the InPlot program (Graph Pad Software, San Diego, CA).

RESULTS AND DISCUSSION

Glycine-activated currents were examined in WT and mutated GlyRs using the whole cell recording mode of the patch-clamp technique. For WT GlyRs, glycine application at a holding potential of −55 mV produced inward currents that showed a half-maximal response (EC_{50}) at 0.029 mM glycine (Table I, Fig. 1). Much higher glycine concentrations were required to elicit currents in R271L and R271Q GlyRs (EC_{50} = 6.7 and 12 mM, respectively) (Table I, Fig. 1), revealing that these mutations caused a 230- and 410-fold reduction in glycine-activated current sensitivity, respectively. The activation and desensitization kinetics of macroscopic currents in R271L and R271Q GlyRs were similar to those of WT GlyRs (Fig. 1*A*), as were the Hill coefficients for activation of glycine-activated currents (Table I). Hence, the startle disease mutations produced a dramatic decrease in glycine sensitivity without appearing to affect either current kinetics or the cooperativity of glycine binding.

The binding properties of the competitive antagonist [^{3}H]strychnine, with the mutant GlyRs, were also examined. The R271L and R271Q GlyRs showed affinities and receptor numbers/cell similar to those for WT GlyRs (Table II, Fig. 2), indicating that these mutations did not affect either the efficiency of expression of GlyRs or their affinity for strychnine. The ability of glycine to displace bound [^{3}H]strychnine was, however, greatly reduced. The glycine inhibition constant for R271L and R271Q GlyRs (K_i = 5.6 and 12 mM, respectively) (Table II) was 56–120-fold higher than that of WT GlyRs (K_i = 0.10 mM) (Table II). Thus, the reduced sensitivity of glycine-activated currents in mutant GlyRs is accompanied by a reduced affinity of glycine binding. That the strychnine sensitivity of the mutant receptors remains unchanged is significant, since binding sites for glycine and strychnine are intimately

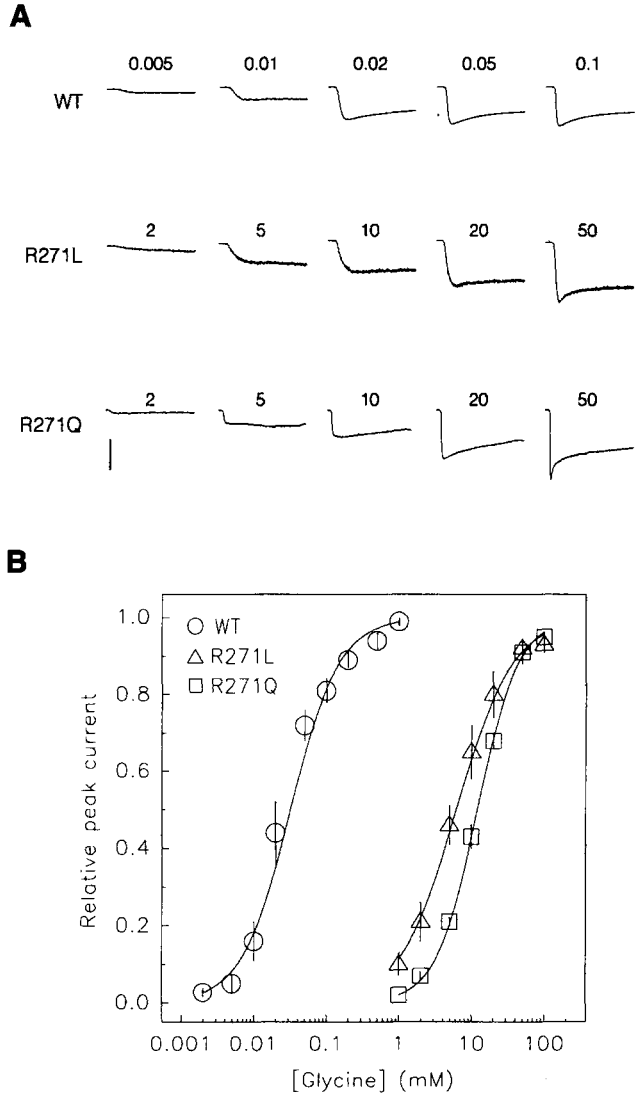

FIG. 1. **Reduced sensitivity of glycine-activated currents in 293 cells expressing R271L and R271Q GlyRs.** *A*, whole cell currents activated by glycine in 293 cells expressing WT, R271L, and R271Q GlyRs. Concentrations of glycine applied (in mM) are listed above each trace. Traces are 10 s in duration. *Scale bar*, 0.5 nA (WT and R271Q GlyRs) or 1 nA (R271L GlyR). *B*, dose-response curves of peak glycine-activated currents. Data represent mean values ± S.E. from 5–6 cells.

TABLE II

Mean B_{max} and K_d values for [^{3}H]strychnine binding isotherms and K_i values for glycine displacement of bound [^{3}H]strychnine in 293 cells expressing WT and mutant GlyRs

The values in parentheses are the -fold changes relative to the values for the WT GlyR. Mean values with S.E. from 3–5 independent determinations are shown.

GlyR	B_{max}	Strychnine K_d	Glycine K_i
	× 10^6 *sites/cell*	*nM*	*mM*
WT	1.3 ± 0.2 (1)	8.1 ± 0.3 (1)	0.10 ± 0.02 (1)
R271L	3.9 ± 0.6 (3)	14 ± 2.2 (1.7)	5.6 ± 0.8 (56)
R271Q	1.7 ± 0.3 (1.3)	9.0 ± 0.9 (1.1)	12 ± 2.2 (120)

associated (4–8). The reductions in glycine sensitivity but not strychnine sensitivity caused by these mutations therefore cannot be due to a general alteration of the structure of the ligand binding region of the GlyR. Other residues exhibiting a similar specificity of action have previously been identified in the GlyR

(4–8) and in the related $GABA_AR$ γ-aminobutyric acid type A receptor (24, 25) and nAChR (reviewed in Ref. 26), but only in the N-terminal extracellular regions of these receptors. Our finding that Arg-271, located at the border of the channel-lining M2 domain of the GlyR, is a highly specific determinant of agonist activation raises the possibility that the ligand binding and ion conducting portions of the GlyR are far more intricately linked than has been previously thought.

The possibility that the channel permeation properties of the GlyR were also altered by mutation of Arg-271 was also investigated. The current-voltage relations of peak glycine-activated currents in the mutant GlyRs were indistinguishable from that of WT GlyRs (Fig. 3). In solutions containing equimolar 153 mM Cl^-, application of saturating concentrations of glycine at various membrane potentials resulted in currents that reversed close to the Cl^- equilibrium potential of 0 mV. Replacement of the bath with a 75 mM Cl^- solution shifted the reversal potential of currents close to the new Cl^- equilibrium potential of +19 mV (Fig. 3*C*), confirming that these currents remained Cl^- selective and that mutation of Arg-271 did not significantly alter the relative cation-anion selectivity. Hence, Arg-271 does not appear to play a major role in determining the macroscopic conductance properties of the GlyR. This was surprising, since this residue has been predicted to affect ion permeation through the receptor (1, 27, 28). Closely corresponding residues in the M2 domain of the nAChR form an outer anionic ring of charge, which influences divalent cation effects on channel conductance (27). Additionally, replacement of arginine residues at positions 252 and 271 with glutamic acid residues confers cation selectivity to GlyR M2 peptides incorporated into lipid bilayers (28). It has been reported recently that the arginine residue at position 252, located at the cytoplasmic margin of the M2 region of the GlyR, is critical for receptor assembly (29). Mutation of this residue to either Glu or Gln prevented the expression of GlyRs on the cell surface. It appears, then, that both Arg-252 and Arg-271 influence GlyR function in manners that extend beyond that of determining ion permeation.

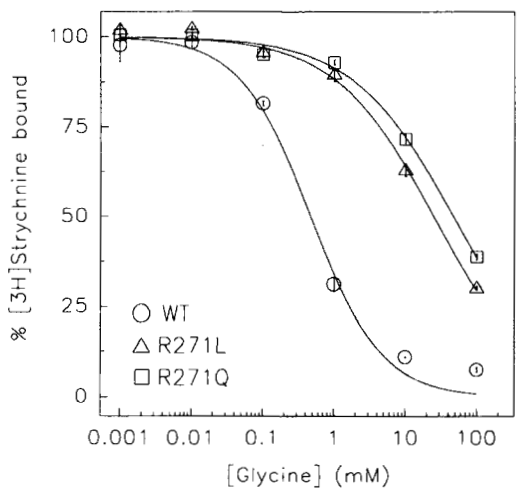

FIG. 2. **Reduced glycine binding affinity of 293 cells expressing R271L and R271Q GlyRs.** Glycine displacement curves of bound [^{3}H]strychnine from 293 cells expressing WT and mutant GlyRs are illustrated. Data represent the mean values ± S.E. of triplicate measurements from a representative experiment.

Our identification of a profound decrease in agonist sensitivity in the mutant GlyRs provides an explanation of the startle disease phenotype, since decreased glycinergic tone in spinal cord interneurons effectively reduces the recurrent and reciprocal inhibitory feedback that modulates firing of motoneurons in reflex arcs. Reduced glycinergic inhibition also underpins the phenotype of the murine autosomal recessive mutation, *spastic*, a form of inherited myoclonus (30). In *spastic*, this reduction is due to the underexpression of GlyRs rather than the impairment of their function (30, 31). It is possible that reduced GlyR expression *in vivo* may also accompany startle disease but this appears unlikely. Our estimates of the number of GlyRs at the cell surface from the predicted numbers of strychnine binding sites (B_{max} values in Table II) indicate that the efficiency of expression of mutant GlyRs was comparable to that of WT GlyRs. Additionally, GlyRs mutated at Arg-271 to

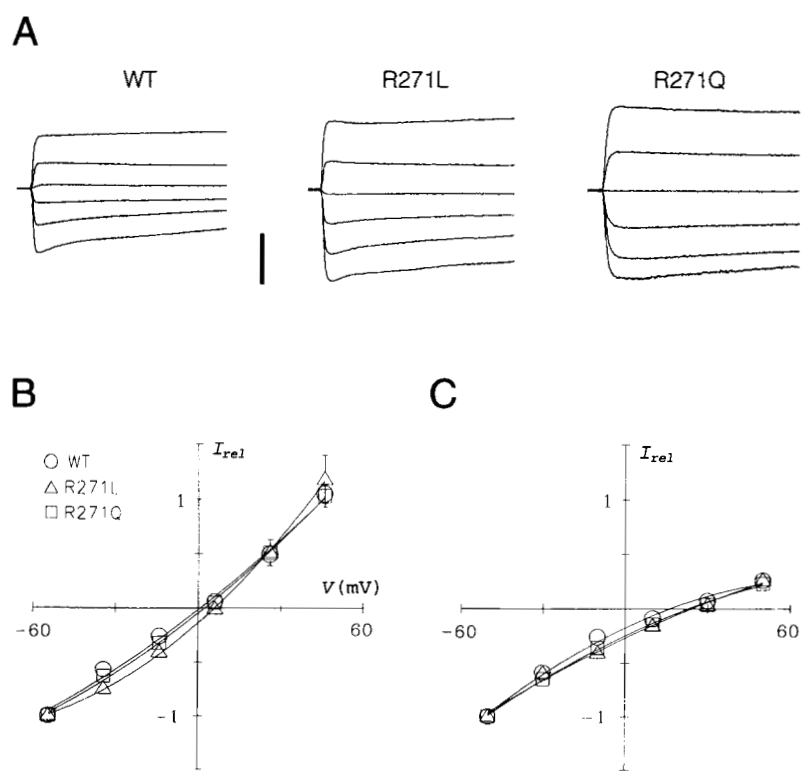

FIG. 3. **Unaltered current-voltage characteristics of 293 cells expressing R271L and R271Q GlyRs.** *A*, whole cell currents activated by 0.1 mM glycine (WT GlyR) or 50 mM glycine (R271L and R271Q GlyRs) at holding potentials of −55 mV (*bottom traces*) to +45 mV (*top traces*) in solutions containing equimolar 153 mM Cl^- are similar in shape and reverse close to 0 mV, the Cl^- equilibrium potential. Currents have been corrected for leakage and voltages for junction potentials. Traces are 5 s in duration. *Scale bar*, 4 nA (WT and R271L GlyRs) or 1 nA (R271Q GlyR). *B*, peak current-voltage relations in solutions containing equimolar 153 mM Cl^-. *C*, for each glycine receptor, subsequent 50% dilution of the bath solution with mannitol resulted in a shift of the reversal potential to near +19 mV, the new Cl^- equilibrium potential. Data represent mean currents standardized to current values at −55 mV with S.E. from 5 cells (*B*) or 3 cells (*C*).

either Glu or Gln assemble at the cell surface in similar numbers to WT GlyRs (29). These mutated receptors were reported to be non-functional, although our data indicate that at least the R271Q GlyRs are capable of forming glycine-gated channels.

Startle disease is rarely lethal (10–13), suggesting that the magnitude of the glycine insensitivity we report in homomeric $\alpha 1$ subunit GlyRs may be less extreme in spinal cord GlyRs due to a dominant negative effect (32). Startle disease heterozygotes, in which both a normal and disease gene are present, will on average, contain only 1.5 mutated $\alpha 1$ subunits in each pentameric GlyR, spinal cord GlyRs being comprised of α and β subunits in a 3:2 stoichiometry (33). Whilst the effects of startle disease mutations are clearly observed using $\alpha 1$ homomeric GlyRs, examination of multi-subunit recombinant GlyRs should further define the startle disease phenotype. A point mutation in the N-terminal extracellular portion of the gene encoding the $\alpha 1$ subunit of the GlyR has also been identified in the murine mutation *spasmodic* (34). The *spasmodic* phenotype resembles both that of startle disease and *spastic*. When expressed in homomeric $\alpha 1$ subunit GlyRs, however, this mutation resulted in only a 6-fold reduction in agonist sensitivity (34). The *spasmodic* mutation, like *spastic*, is an autosomal recessive mutation. Therefore, all $\alpha 1$ subunits present in *spasmodic* spinal cord GlyRs will be of the disease type. Thus, the reduced agonist sensitivity of *spasmodic* and startle disease GlyRs may be more comparable *in vivo*. In summary, the startle disease mutations identified at Arg-271 have revealed an unexpected structural determinant of GlyR function. Analysis of other startle disease mutations may provide further important insights into the mechanisms of action of the ligand-gated ion channel receptor superfamily.

Acknowledgments—We thank Stephen Ryan, Rita Shiang, John Wasmuth, and Peter O'Connell for their enthusiastic support of this study, Trevor Woodage for valuable discussions, and Marjorie Liu, Yvonne Hort, Vikki Falls, and Cheryl Handford for excellent technical assistance.

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